The Hansenula polymorpha peroxisomal targeting signal 1 receptor, Pex5p, functions as a tetramer

Moscicka, Katarzyna B.; Klompmaker, Sandra H.; Wang, Dongyuan; van der Klei, Ida J.; Boekema, Egbert J.

Published in:
FEBS Letters

DOI:
10.1016/j.febslet.2007.03.061

Publication date:
2007

FEBS Letters 581 (2007) 1758–1762

The *Hansenula polymorpha* peroxisomal targeting signal 1 receptor, Pex5p, functions as a tetramer

Katarzyna B. Moscicka[a], Sandra H. Klompmaker[b], Dongyuan Wang[b], Ida J. van der Klei[b], Egbert J. Boekema[a,*]

[a] *Department of Biophysical Chemistry, GBB, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*
[b] *Department of Eukaryotic Microbiology, GBB, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

Received 22 December 2006; revised 14 March 2007; accepted 18 March 2007

Available online 2 April 2007

Edited by Ulrike Kutay

Abstract **We have studied *Hansenula polymorpha* Pex5p and Pex20p, peroxins involved in peroxisomal matrix protein import. In vitro binding experiments suggested that *H. polymorpha* Pex5p and Pex20p physically interact. We used single particle electron microscopy (EM) to analyze the structure of purified Pex5p and its possible association with Pex20p. Upon addition of Pex20p, a multimeric Pex20p complex was observed to be associated to the periphery of the Pex5p tetramer. In this Pex5p–Pex20p complex, the conformation of tetrameric Pex5p had changed from a closed conformation with a diameter of 115 Å into an open conformation of 134 Å. EM also indicated that the Pex5p–Pex20p complex was capable to bind native, folded catalase, a peroxisomal PTS1 protein. This suggests that the Pex5p–Pex20p complex may be functional as receptor complex.**

Keywords: Pex5p; Peroxisome; Membrane transport; Electron microscopy; *Hansenula polymorpha*

1. Introduction

Peroxisomal matrix proteins are synthesized on free polyribosomes and directed to the organelle by specific peroxisomal targeting signals (PTSs). The routing of most matrix proteins depends on one of the two conserved PTSs, designated PTS1 and PTS2, which are recognized by the receptor proteins Pex5p or Pex7p, respectively.

The *PEX5* gene encodes the PTS1 receptor, Pex5p, which interacts with the PTS1 signal via a series of tetratricopeptide repeats (TPRs) within its C terminus. A crystal structure has been determined of a 41 kDa fragment of human Pex5p that includes six TPR motifs in complex with a small peptide containing a PTS1 sequence [1,2] or the sterol carrier protein [3]. This structure reveals the molecular basis for PTS1 recognition which is mostly formed by two clusters of three TPRs almost completely surrounding the PTS1-peptide.

However, whether or not Pex5p functions as an oligomer, is still a matter of debate. Gel filtration chromatography and electron microscopy studies indicated that human Pex5p (HsPex5p) is a homotetramer [4]. Fluorescence spectroscopy studies on Pex5p of the yeast *Hansenula polymorpha* (HpPex5p) indicated that HpPex5p also forms oligomers [5]. Moreover, HpPex5p oligomers were shown to bind PTS1 containing synthetic peptides, suggesting that this is indeed the conformation of a functional PTS1 receptor [6]. On the other hand, recent studies on human Pex5p (HsPex5p) using sucrose density centrifugation revealed that HsPex5p is monomeric [7]. The behavior of HsPex5p in gel filtration chromatography, which indicated a high molecular weight of the native protein, was suggested to result from a non-globular shape of the protein. Studies on the N-terminal domain of HsPex5p pointed to an unfolded pre-molten globule-like structure, which may contribute to a non-globular shape of full length HsPex5p [8].

Pex7p is the receptor for the PTS2 signal. In higher eukaryotes (plants, mammals), Pex7p associates with Pex5p during peroxisomal protein import. In lower eukaryotes (yeasts, fungi) Pex7p binds to Pex20p (or Pex18p and Pex21p in *Saaccharomyces cerevisiae*; for a review see [9]). For PTS2 protein import Pex20p's most likely fulfill a similar function as Pex5p in higher eukaryotes. Indeed, the N-terminal half of Pex5p's and Pex20p's share a few conserved domains and show similar dynamics during import [9,10]. Like Pex5p also Pex20p has been reported to form oligomers [11].

Our understanding of the structure of the peroxins involved in peroxisomal protein translocation is still limited. For initial characterization of such complexes single particle electron microscopy (EM) is a well-established technique to obtain information at a resolution of 10–20 Å [12,13]. It is attractive for samples of mixed complexes with a mass above about 200 kDa, because the statistical analysis and classification procedures used are effective in sorting of (slightly) different projection views originating from different conformations or subunit compositions. In this study, the projection structures of HpPex5p and HpPex5p–HpPex20p complexes were investigated by single particle electron microscopy. The analysis shows that HpPex5p is a tetramer and that HpPex20p is able to induce a major conformational change leading to a rather open space in the centre of the HpPex5p tetramer. In a successive set of experiments, we show that HpPex5p–HpPex20p complexes are able to bind folded copies of tetrameric catalase at the periphery. Since catalase is one of the major peroxisomal proteins this indicates that such HpPex5p–HpPex20p-catalase complexes are functional as receptor complex.

*Corresponding author. Fax: +31 503634800.
E-mail address: e.j.boekema@rug.nl (E.J. Boekema).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.03.061

2. Materials and methods

2.1. Organisms and growth

The *Hansenula polymorpha* strains used in this study are wild type (WT), *pex5*, *pex7* and *pex20*. Yeast cells were grown in a carbon-limited chemostat culture at 37 °C using 0.25% glucose as carbon source and 0.2% choline as nitrogen source at a dilution rate of 0.1 h^{-1}. Cells were harvested from steady state cultures. The levels of Pex5p and Pex20p were analyzed by western blotting using total cell lysates.

2.2. Expression and purification

Escherichia coli M15 cells containing plasmids pREP4 and pQE60–Pex20p-His_8 [11] or pQE60–Pex5p-His_6 [6] were grown as described before. Expression of the *PEX5* or *PEX20* genes was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside as detailed before [6,11]. Cells were harvested by centrifugation. All subsequent steps were carried out at 4 °C. Pex5p was purified as detailed [6]. To isolate the Pex5p–Pex20p complex, Pex5p-His_6 and Pex20p-His_8 were isolated by affinity chromatography using Ni-NTA (Qiagen). Cell pellets were resuspended in 40 ml buffer A (50 mM phosphate buffer, pH 7.4, 300 mM NaCl, 1% Tween 20, 10% glycerol, 0.2 mM β-mercaptoethanol, 1 mM phenylmethyl sulfonyl fluoride, 1 mM sodium azide, 5 mM sodium fluoride, and Complete™ (Roche, Almere, The Netherlands). Cells were disrupted using a French Press. The lysates were centrifuged (20 min, $10\,000 \times g$), and supernatants incubated for 1 h with Ni-NTA (Qiagen, 500 mg protein/ml resin). Subsequently, the resin was extensively washed with buffer B (50 mM phosphate buffer, pH 7.4, 100 mM NaCl) containing increasing concentrations of imidazole (up to 40 mM). Bound proteins were eluted with buffer B containing 200 mM imidazole. Elution fractions were analyzed by SDS–PAGE followed by Coomassie brilliant blue staining and Western blotting using antibodies raised against Pex5p or Pex20p. Fractions highly enriched in Pex5p-His_6or Pex20p-His_8, respectively were pooled. Equal portions of these fractions were mixed and incubated for one hour on ice. Then, the Pex5p-His_6–Pex20p-His_8 mixture was subjected to gel filtration chromatography (Superose 12) using 50 mM potassium phosphate buffer, pH 7.4 as running buffer. The elution fractions were analyzed by SDS–PAGE followed by Coomassie brilliant blue staining and Western blotting using anti-Pex5p and anti-Pex20p antibodies. The first fractions that eluted from the column and contained both Pex5p-His_6 and Pex20p-His_8 were used for electron microscopy.

Gel filtration experiments to estimate the mass of Pex5p and Pex5p–Pex20p complexes were performed on a Superose 6 size exclusion column on an AKTA FPLC system, equipped with a UV detector with catalase, ferritin, thyroglobulin as marker proteins.

2.3. In vitro binding studies

Pex5p-His_6 and Pex20p-His_8 were produced in *E. coli* and purified using Ni-NTA agarose as detailed above. Fractions containing Pex20p or Pex5p were diluted 10 times using buffer A and incubated with Ni-NTA agarose for 1 h at 4 °C. Methanol grown *H. polymorpha* wild type or *pex7* cells were disrupted using glass beads. Cell homogenates were centrifuged at 4 °C for 5 min at $20\,000 \times g$ to remove unbroken cells and cell debris. Supernatants were loaded onto the Ni-NTA agarose containing either Pex20p-His_8 or Pex5p-His_6 and incubated for 1 h at 4 °C with continuous rotation. After extensive washing with buffer B containing increasing concentrations of imidazole (from 10 mM up to 40 mM), Pex20p-His_8 or Pex5p-His_6 together with the bound proteins were eluted with buffer B containing 250 mM imidazole. The elution fractions were analyzed by Western blotting.

2.4. Preparation Pex5p-Pex20p complexes with catalase

Crystalline catalase from bovine liver (from Sigma-Aldrich) at a stock concentration of 20 mg/ml was diluted with water to the final concentration of 33 μg/ml. Pex5p–Pex20p (5 μl) complexes from the best fractions were incubated with 1 μl catalase for 1 h at room temperature and immediately prepared for EM.

2.5. Electron microscopy and single-particle analysis

Negatively stained Pex5p, Pex5p–Pex20p and Pex5p–Pex20p complexes mixed with catalase were prepared with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM12 FEG electron microscope operated at 120 kV. Images were recorded with a Gatan 4 K slow-scan CCD camera at 80 000× magnification with a pixel size (after binning the images) of 3.75 Å at the specimen level, with "GRACE" software for semi-automated specimen selection and data acquisition. About 6000 images were recorded and about 7520 single particle projections from Pex5p, 6071 projections from Pex5-20 complexes, 1300 from Pex5-20p-catalase and 5800 single catalase projections were selected, respectively. Single-particle analysis was performed with the Groningen Image Processing ("GRIP") software package on a PC cluster. Selected single-particle projections (128×128 pixel frame) were aligned by a multireference alignment and reference-free alignment procedures according to [13,14]. Next, particles were subjected to multivariate statistical analysis, followed by hierarchical ascendant classification (HAC) [13]. The resolution of the class averages was measured by Fourier Ring Correlation according to [15]. After several cycles of multireference alignments, statistical analysis and classification the best projections from each set were averaged.

3. Results and discussion

We recently performed a detailed comparative study on *H. polymorpha pex* mutants, which were identically grown in chemostat cultures [16] (Koek and Van der Klei, unpublished results). Unexpectedly, in samples of steady state cultures we observed that the levels of HpPex5p were reduced in cells lacking Pex20p (*H. polymorpha pex20* cells), whereas HpPex20p levels were lower in the absence of Pex5p (in *H. polymorpha pex5*), as shown in Fig. 1. This indicates that HpPex5p and HpPex20p may stabilize each other in vivo. To analyze whether this apparent stabilization is related to a physical interaction between both proteins, we performed in vitro interaction studies. Crude extracts of *H. polymorpha* wild type cells were loaded onto columns containing either immobilized His-tagged HpPex5p or HpPex20p, purified from *E. coli*. As shown in Fig. 2A (lane 2), Pex20p from the *H. polymorpha* extract co-eluted with Pex5p-His_6. The cytosolic enzyme pyruvate carboxylase (Pyc1p), used as a negative control, did not co-elute with Pex5p-His_6. Moreover, Pex20p was not detected in the eluate when an empty column was used (Fig. 2A, lane 1). In a reverse approach, using a column containing Pex20p-His_8, Pex5p from the *H. polymorpha* extract co-eluted with Pex20p-His_8 (Fig. 2B, Lane 2). Again, the Pyc1p control protein did not co-elute with Pex20p-His_8 and no Pex5p was present in the elution fraction when an empty column was used

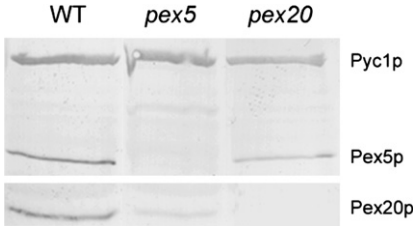

Fig. 1. Pex5p and Pex20p levels in cells of *H. polymorpha* WT and *pex* mutants. Shown are steady-state levels of Pex5p and Pex20p in WT and *pex5* and *pex20* mutants grown in chemostat cultures on glucose/choline. Pex5p and Pex20p levels were analyzed by Western blotting using specific antibodies against Pex5p and Pex20p, respectively. Pex5p and Pex20p are both evident in WT cells. In *pex5* cells Pex5p is absent as expected. However, in addition the level of Pex20p is strongly reduced in *pex5* cells. Similarly, Pex20p is absent in *pex20* cells, but Pex5p levels are slightly reduced. Equal amounts of protein were loaded per lane. The cytosolic protein pyruvate carboxylase (Pyc1p) was included as a loading control.

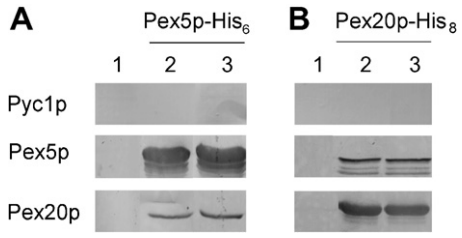

Fig. 2. *H. polymorpha* Pex5p and Pex20p physically interact in vitro. (A,B) Pex5p-His_6 (lanes 2 and 3 in (A))or Pex20p-His_8 (lanes 2 and 3 in (B)), produced in *E. coli*, were immobilized on Ni-NTA columns. Empty Ni-NTA columns (lane 1) were used as controls. Crude extracts of *H. polymorpha* WT (lanes 1 and 2) or *pex7* cells (lane 3) were loaded onto the columns. After washing with buffer, the His-tagged proteins together with bound proteins were eluted using imidazole. Equal portions of the elution fractions were loaded onto an SDS–PAGE gel and subjected to Western blot analysis using antibodies against pyruvate carboxylase (Pyc1p), Pex5p or Pex20p as indicated.

(Fig. 2B, lane 1). Co-elution of Pex5p with Pex20p and vice versa was also observed when crude extracts of *H. polymorpha* cells were used in which the PTS2 receptor Pex7p was absent (*H. polymorpha pex7* cells), indicating Pex7p is not required for association of Pex5p with Pex20p (Fig. 2A, B, lane 3).

In order to study the structure of Pex5p and its association with Pex20p, both proteins were produced in *E. coli*, purified (see Fig. 3A and B) and analyzed by electron microscopy (EM). EM of negatively stained purified Pex5p complexes indicated high numbers of tetrameric particles in one type of projection (not shown). In specimens of the Pex5p–Pex20p mixture small numbers of the same type of closed tetramer were observed (black arrowhead, Fig. 4), but the vast majority of the tetramers was slightly expanded. Many of these open tetramers had one and occasionally two or three globular masses attached (white arrowheads, Fig. 4). A total number of about 14000 projections of the Pex5p, Pex5p–Pex20p and

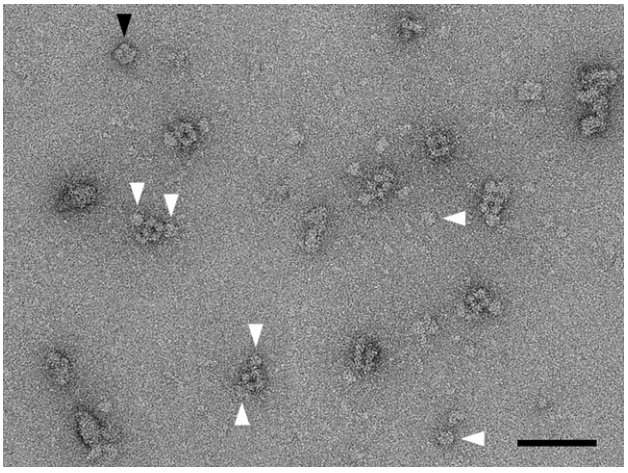

Fig. 4. Electron micrograph of negatively stained Pex5p–Pex20p complexes. The black arrowhead points to a Pex5p tetramer in the closed conformation; the white arrowheads indicate Pex20p in the open conformation, either attached to Pex5p or as single complexes. The bar indicates 50 nm.

Pex20p particles were analyzed by single particle electron microscopy. Analysis shows that the purified Pex5p forms a closed tetramer with a diameter of 115 Å (Fig. 5A). This four-fold symmetrical top-view is almost the only type of projection and side-views were very rare. Apparently molecules cannot attach to the carbon support film in any stable side-view position, which indicates that the tetramer should be rather flat (see also below for the shape of catalase). Thus it is very likely that each 1/4 of the tetramer is compatible to a monomer of Pex5p, if compared to other multi-subunit complexes with known composition and exact mass. With an estimated height of 50–70 Å, a diameter of 115 Å and a mass of a tetramer of 4×67 kDa, the tetramers are for instance compatible with

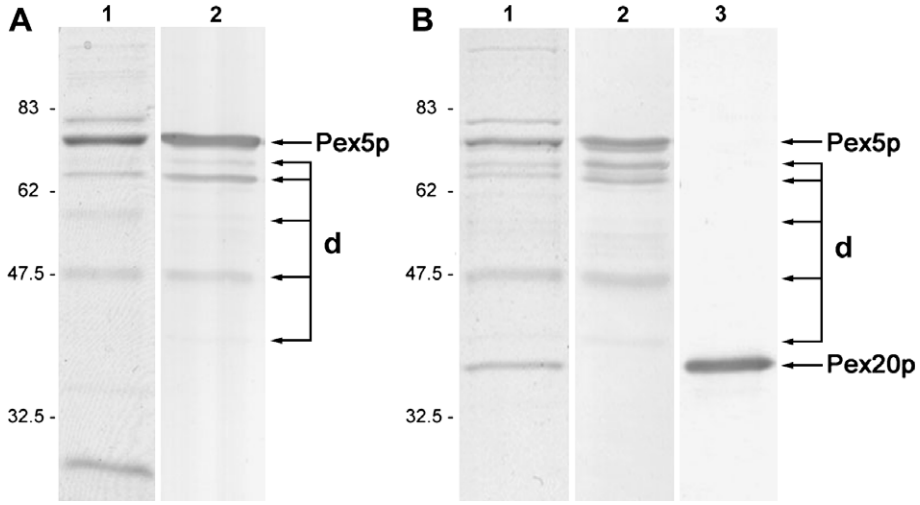

Fig. 3. Purification of Pex5p-His_6 and Pex5p-His_6/Pex20p-His_8 complexes. *H. polymorpha* Pex5p-His_6 and Pex20p-His_8 were produced in *E. coli*. (A) Lane 1 shows a Coomassie brilliant blue stained gel of the protein fraction containing HpPex5p. The major protein band at ≈67 kDa represents Pex5p, as is evident from the Western blot decorated with anti-Pex5p antibodies (lane 2). The higher protein band of approx. 80 kDa visible in lane 1 represents *Escherichia coli* hsp70 as was evident from mass spectrometry analysis (data not shown). The few minor bands of lower molecular weight most likely represent Pex5p degradation products as they are recognized by the anti-Pex5p antibodies (lane 2, indicated by d). (B) A gel of a Pex5p and Pex20p containing fraction obtained upon gel filtration of a mixture of Pex5p and Pex20p. Lane 1 shows a gel stained with Coomassie brilliant blue. Lane 2 shows the corresponding Western blot decorated with anti-Pex5p antibodies and lane 3 the corresponding blot stained with anti-Pex20p antibodies. The Pex5p degradation bands are indicated by d.

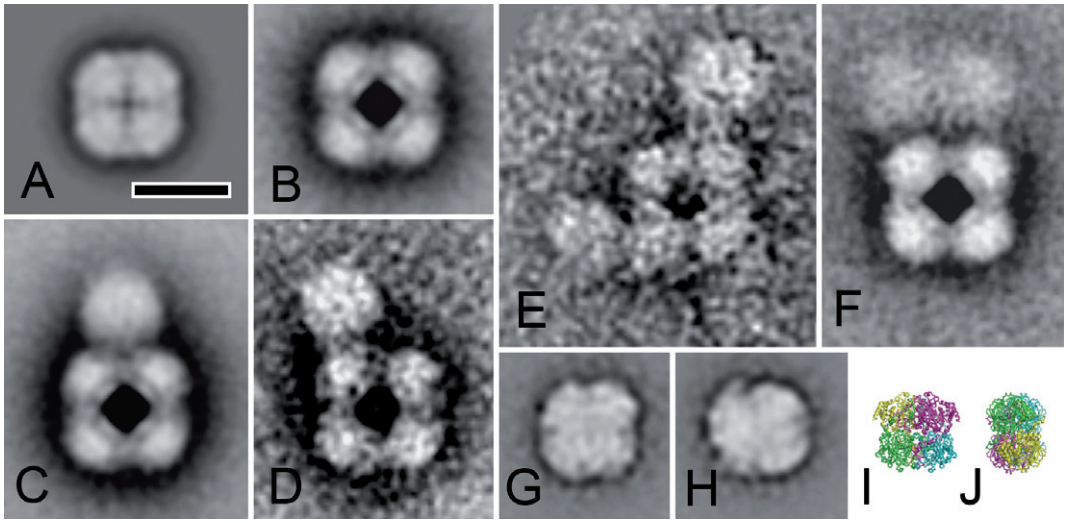

Fig. 5. Single particle image analysis of Pex5p, Pex5p–Pex20p and Pex5p–Pex20p-catalase complexes. (A) Average projection map of purified Pex5p in the closed conformation. (B) Average projection map of purified Pex5p in the open conformation. (C) Main view of the Pex5p–Pex20p complex. (D) Average map of a small class of Pex5–Pex20p complexes in which the upper Pex20p multimer is displaced. (E) Another class of Pex5p–Pex20p complexes in which a Pex20p multimer second multimer is binding to the left side of the Pex5p tetramer. (F) Main class of Pex5p–Pex20p-catalase complexes (G,H) main views of purified catalase tetramers. (I) High-resolution catalase X-ray model in a position similar to the EM projection of image G, in which it is slightly tilted out of its fourfold symmetrical view. (J) Side-view of the X-ray model [20] in which two monomers are almost in overlap with two others. Fourfold symmetry was imposed on images of (A) and (B) after completion of analysis. The bar equals 10 nm.

the $\alpha_3\beta_3$ F_1 ATP synthase subcomplex ($6\times\sim55$ kDa; 110 Å diameter; height ~85 Å) [17]. Indeed, the behaviour of HpPex5p in gel filtration chromatography (not shown) also suggests that the protein is not monomeric, because it eluted in the same range as the marker proteins catalase and ferritin (440 kDa). However, it is in general impossible to draw firm conclusions from size exclusion chromotography about the exact number of copies of a multimeric protein such as Pex5p.

The EM analyses also revealed that in the Pex5p–Pex20p sample about 75% of the Pex5p tetramers is complexed with a globular mass of about 80 Å in 1–3 copies (Fig. 5C–E). Additional EM experiments with purified Pex20p revealed similar-shaped 80 Å globular densities (not shown), indicating that these globular densities are composed of a Pex20p multimer. In the majority of the Pex5p tetramers the Pex20p multimer binds at the interface between two monomers (Fig. 5C), in small subsets it is displaced (Fig. 5D and E). Previous fluorescence correlation spectroscopy experiments have indicated that the 35 kDa Pex20p protein forms multimers with a mass of about 200 kDa, probably hexamers [11]. Unfortunately, the data did not resolve the inner features of Pex20p, probably due to multiple orientations. The resolution in the EM maps of Fig. 5A and B is about 20 Å, but in the one of Fig. 5D only about 30 Å. However, the binding of most of the Pex20p multimers to the Pex5p tetramer is rather specific (Fig. 5C). Binding of Pex20p leads to an unexpected increase in the diameter of the tetramers from 115 Å to 134 Å and a pronounced change of the overall conformation. The increase was observed in 98% of the tetramers from the Pex5p–Pex20p batch, even if no Pex20p was bound (Fig. 5B), which was the case in 25% of the tetramers. Probably, such tetramers had lost the Pex20p multimer after the conformational change upon preparation for EM, because in 5% of Pex5p–Pex20p complexes the Pex20p multimer is almost detached (see upper multimer in Fig. 5E). About 15% of the Pex5p tetramers has a second or third Pex20p complex attached (Fig. 5E and not shown).

The Pex5p–Pex20p complexes eluted from the gelfiltration column before the marker protein thyroglobulin (669 kDa), confirming that both proteins are present in complexes that are larger than the Pex5p tetramers.

Subsequently, we aimed to study the binding of potential cargo to the Pex5p–Pex20p complexes. To this end, the PTS1 protein catalase was used. Single catalase tetramers could be averaged with reasonable detail after statistical analysis and classification (Fig. 5G and H). Fig. 4G shows a class-sum which matches the high-resolution X-ray structure of catalase of Fig. 5I. Side-views of the catalase tetramer, such as presented in Fig. 5J, were not observed. Samples of Pex5p–Pex20p complexes incubated with catalase were inspected for larger complexes. Small numbers of Pex5p–Pex20p-catalase complexes could be observed. Classification indicates only binding of catalase at the periphery of the Pex5p tetramers, preferentially next to a Pex20p multimer. An averaged sum of the best 153 complexes shows, however, a fuzzy appearance of the Pex20p multimer (left) and the catalase tetramer (Fig. 5F). The assumed catalase density has the expected overall dimensions deduced from the purified catalase, but can not be assigned more positively. However, we do not consider the second density to be another Pex20p multimer, because we did not find any significant numbers of such particles with two Pex20p multimers at one side of the Pex5p tetramer before admission of catalase. The blurred circumference of the catalase in Fig. 5F strongly suggests that it is bound at slightly different positions within the Pex5p–Pex20p-catalase complexes. Hence no further conclusions on the precise binding of catalase (in top- or side view orientation) can be drawn.

Our data convincingly demonstrate that *H. polymorpha* Pex5p is arranged as a tetramer. Also, the conformational change of the Pex5p tetramer associated with Pex20p binding is clearly resolved. The widening of the tetramer most likely occurs via a major rearrangement of protein domains. This rearrangement could lead to replacement of the hinge region,

which has been assigned in the atomic structure [1] and which could lead to a movement of TPRs1–3 in respect to TPRs 4–6. It would be tempting to fit the atomic X-ray structure into the EM map of Pex5p. However, the fact that the X-ray structure only contains a fragment of Pex5p and that the EM map has a much lower (20 Å) resolution makes it impossible to relate them at the current stage. This prevents speculating about domains of Pex5p which might be important for cargo transport at the atomic level. Further research will be necessary to clarify if the open centre has this specific function, or if it is a secondary effect of a conformational change in the centre of each Pex5p monomer. Nevertheless, the lower-resolution EM data clearly indicate that the peripheral binding of Pex20p and catalase to the Pex5p tetramer appears to be relevant. Since also folded proteins can be translocated over the peroxisomal membrane [18], we speculate that the Pex5p–Pex20p-catalase complex is a structural entity that can be transported over the membrane. This would suggest that – like in higher eukaryotes – import of PTS1 and PTS2 proteins also converges at the level of the receptor complexes in lower eukaryotes, as recently suggested [9].

The *H. polymorpha* Pex5p–Pex20p complex is one of the few complexes involved in the transport of folded proteins which has been structurally characterized. Another system is the twin-arginine (Tat) translocation system of bacteria, but this system has no structural similarity to the Pex5p–Pex20p complex [19].

Acknowledgements: Wilma Bergsma, Wilko Keegstra and Gert Oostergetel for help with electron microscopy, Anne Koek for growing yeast cells in chemostat cultures, Wim Huibers for performing mass spectrometry and Marten Veenhuis for discussions and critical reading of the manuscript. E.J.B. acknowledges financial support from the Netherlands Proteomics Centre (NPC). D.W. and I.vd.K are supported by a grant of the Earth and Life foundation (ALW), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

References

[1] Gatto, G.J., Geisbrecht, B.V., Gould, S.J. and Berg, J.M. (2000) Peroxisomal targeting signal-1 recognition by the TPR domains of human PEX5. Nat. Struct. Biol. 7 (12), 1091–1095.
[2] Gatto, G.J., Maynard, E.L., Guerrerio, A.L., Geisbrecht, B.V., Gould, S.J. and Berg, J.M. (2003) Correlating structure and affinity for PEX5:PTS1 complexes. Biochemistry 42 (6), 1660–1666.
[3] Stanley, W.A., Filipp, F.V., Kursula, P., Schuller, N., Erdmann, R., Schliebs, W., Sattler, M. and Wilmanns, M. (2006) Recognition of a functional peroxisome type 1 target by the dynamic import receptor pex5p. Mol. Cell. 24 (5), 653–663.
[4] Schliebs, W., Saidowsky, J., Agianian, B., Dodt, G., Herberg, F.W. and Kunau, W.H. (1999) Recombinant human peroxisomal targeting signal receptor PEX5. J. Biol. Chem. 274 (9), 5666–5673.
[5] Boteva, R., Koek, A., Visser, N.V., Visser, A.J., Krieger, E., Zlateva, T., Veenhuis, M. and Van der Klei, I.J. (2003) Fluorescence analysis of the *Hansenula polymorpha* peroxisomal targeting signal-1 receptor, Pex5p. Eur. J. Biochem. 270 (21), 4332–4338.
[6] Wang, D., Visser, N.V., Veenhuis, M. and van der Klei, I.J. (2003) Physical interactions of the peroxisomal targeting signal 1 receptor Pex5p, studied by fluorescence correlations spectroscopy. J. Biol. Chem. 278 (44), 43340–43345.
[7] Costa-Rodrigues, J., Carvalho, A.F., Fransen, M., Hambruch, E., Schliebs, W., Sa-Miranda, C. and Azevedo, J.E. (2005) Pex5p, the peroxisomal cycling receptor, is a monomeric non-globular protein. J. Biol. Chem. 280 (26), 24404–24411.
[8] Carvalho, A.F., Costa-Rodrigues, J., Correia, I., Costa Pessoa, J., Faria, T.Q., Martins, C.L., Fransen, M., Sa-Miranda, C. and Azevedo, J.E. (2006) The N-terminal half of the peroxisomal cycling receptor Pex5p is a natively unfolded domain. J. Mol. Biol. 356 (4), 864–875.
[9] Kiel, J.A., Veenhuis, M. and van der Klei, I.J. (2006) PEX genes in fungal genomes: common, rare or redundant. Traffic 7 (10), 1291–1303.
[10] Leon, S., Zhang, L., McDonald, W.H., Yates, J., Cregg, J.M. and Subramani, S. (2006) Dynamics of the peroxisomal import cycle of PpPex20p: ubiquitin-dependent localization and regulation. J. Cell Biol. 172 (1), 67–78.
[11] Otzen, M., Wang, D., Lunenborg, M.G.J. and van der Klei, I.J. (2005) *Hansenula polymorpha* Pex20p is an oligomer that binds the peroxisomal targeting signal 2 (PTS2). J. Cell. Sci. 118 (15), 3409–3418.
[12] Frank, J. (2002) Single-particle imaging of macromolecules by cryoelectron microscopy. Annu. Rev. Biophys. Biomol. Struct. 31, 309–319.
[13] Van Heel, M., Gowen, B., Matadeen, R., Orlova, E.V., Finn, R., Pape, T., Cohen, D., Stark, H., Schmidt, R., Schatz, M. and Patwardhan, A. (2000) Single-particle electron cryo-microscopy: towards atomic resolution. Quat. Rev. Biophys. 33 (4), 307–369.
[14] Penczek, P., Radermacher, M. and Frank, J. (1992) Three dimensional reconstruction of single particles embedded in ice. Ultramicroscope 40 (1), 33–53.
[15] van Heel, M. (1987) Similarity measures between images. Ultramicroscope 21 (1), 95–100.
[16] Koek, A. (2006) Peroxisomes in *Hansenula polymorpha*. Ph.D. Thesis University of Groningen, The Netherlands.
[17] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J.E. (1994) Structure at 2.8-Angstrom resolution of F1-ATPase from bovine heart-mitochondria. Nature 370 (6491), 621–628.
[18] Leon, S., Goodman, J.M. and Subramani, S. (2006) Uniqueness of the mechanism of protein import into the peroxisome matrix: transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors. Biochim. Biophys. Acta 1762 (12), 1552–1564.
[19] Oates, J., Mathers, J., Mangels, D., Kühlbrandt, W., Robinson, C. and Model, K. (2003) Consensus structural features of purified bacterial TatABC complexes. J. Mol. Biol. 330 (2), 277–286.
[20] Sugadev, R., Balasundaresan, D., Ponnuswamy, M.N., Kumaran, D., Swaminathan, S., Sekar, K. The crystal structure of bovine liver catalase. Unpublished, PDB entry: 1TGU.